

**Human CD3-specific antibody with immunosuppressive properties**

The present invention relates to mono- and multivalent scFv-antibodies comprising the binding sites specific for the human T cell marker CD3. The antibodies of the invention are strongly immunosuppressive and do not cause a significant release of cytokines. The present invention also relates to polynucleotides encoding said antibodies as well as vectors comprising said polynucleotides, host cells transformed therewith and their use in the production of said antibodies. Finally, the present invention relates to compositions, preferably pharmaceutical compositions, comprising any of the above mentioned polynucleotides, antibodies or vectors. The pharmaceutical compositions are useful for immunotherapy, preferably against acute transplant rejections.

OKT3, a murine IgG2a mAb directed against the  $\epsilon$ -chain of the CD3 complex on human T lymphocytes (Salmeron et al., J. Immunol. 147 (1991), 3047-3052) and produced by a hybridoma with the ATCC deposit number of CRL 8001 is used to prevent tissue rejection after renal and hepatic transplantation, and provides an alternative treatment for transplant rejections that are unresponsive to corticosteroids. In vivo, administration of OKT3 induces a dramatic decrease in the number of circulating CD3<sup>+</sup> cells as it down-modulates the T-cell receptor (TCR). However, adverse effects can occur during the first days of treatment. Chills and fever often follow the administration of OKT3 and patients occasionally suffer from nausea, vomiting, diarrhea, dyspnea, wheezing, and sterile meningitis. Many of these side effects have been attributed to the release of cytokines, especially from T cells. After a more prolonged period of use, many patients develop a human anti-mouse antibody (HAMA) response.

Binding of OKT3 alone is insufficient to trigger T cells. Proliferation of T cells which induces the release of cytokines like IL-2, IL-6, TNF- $\alpha$  and IFN- $\gamma$  results from cross-linking of T cells and FcR-bearing cells. Human IgG Fc receptors (Fc $\gamma$ RI, Fc $\gamma$ RII, Fc $\gamma$ RIII) are distributed on human monocytes/macrophages, B lymphocytes, NK cells and granulocytes. They all bind to the C<sub>H</sub>2 region of both mouse and human IgG, differing in their affinity. The immunogenicity of such anti-CD3 Ab has been reduced by using chimeric antibodies made from the variable domains of a mouse mAb and the constant regions of a human Ab. To reduce binding to Fc receptors, Fc domains from particular classes of human IgG have been employed or mutations have been introduced into the Fc domain in the parts that bind to the Fc receptors. However, interactions of the Fc domains cannot be completely abrogated and the efficacy of the immunosuppressive activity was not increased.

Thus, the technical problem underlying the present invention was to provide means more suitable for preventing allograft rejection that overcome the disadvantages of the means of the prior art.

The solution of the said technical problem is achieved by providing the embodiments characterized in the claims. Antibodies have been constructed that are more efficient in suppressing T cell activation and proliferation by down-regulating the CD3 molecule but that do not cause a large release of cytokines, thus avoiding many of the unpleasant side-effects. These antibodies only comprise the variable immunoglobulin domains, so called F<sub>v</sub> modules by means of which undesired immune responses can be avoided. The F<sub>v</sub> module is formed by association of the immunoglobulin heavy and light chain variable domains, V<sub>H</sub> and V<sub>L</sub>, respectively. Preferred

embodiments of these antibodies are based only on the variable domains of the OKT3 antibody, but contain a serine instead of a cysteine at position H100A of the heavy chain (according to the Kabat numbering system). This mutation has previously been shown to improve the stability of the single chain Fv molecule (Kipriyanov et al., Protein Engineering 10 (1997), 445-453). Surprisingly, such antibodies, and in particular a bivalent antibody in a so-called diabody format, had a much greater immunosuppressive effect as measured by CD3 downregulation and inhibition of T cell proliferation in a mixed lymphocyte reaction (MLR) than the original parental OKT3 antibody and, in contrast to the parental OKT3, caused no significant release of the cytokines IFN- $\alpha$  and IL-2.

#### **Brief description of the drawings**

##### FIGURE 1: Schematic representation of mono- and multivalent single chain Fv-antibody constructs

Diabody: non-covalent scFv dimer; scDb: single chain diabody; scFv: single chain Fv fragment; (scFv)<sub>2</sub>: scFv-scFv dimer. The antibody V<sub>H</sub> and V<sub>L</sub> domains are shown as black and gray ovals, respectively.

##### FIGURE 2: Expression cassettes for anti-CD3 scFv constructs

His<sub>6</sub>: six C-terminal histidine residues; L: short peptide linker (the amino acid sequence is shown in bold) connecting the V<sub>H</sub> and V<sub>L</sub> domains; leader, bacterial leader sequence (e.g. PelB leader) for secretion of recombinant product into periplasm; rbs, ribosome binding site; Stop: stop codon (TAA); V<sub>H</sub> and V<sub>L</sub>: variable regions of the heavy and light chains specific to human CD3. Four C-terminal amino acids of V<sub>H</sub> domain and four N-terminal amino acids of the V<sub>L</sub> domain are underlined.

FIGURE 3: Diagram of the expression plasmid pSKK3-scFv6\_OKT3

bla: gene of beta-lactamase responsible for ampicillin resistance; bp: base pairs; CDR-H1, CDR-H2 and CDR-H3: sequence encoding the complementarity determining regions (CDR) 1-3 of the heavy chain; CDR-L1, CDR-L1, CDR-L2 and CDR-L3: sequence encoding the complementarity determining regions (CDR) 1-3 of the light chain; CH1-L6 linker: sequence which encodes the 6 amino acid peptide Ser-Ala-Lys-Thr-Thr-Pro connecting the  $V_H$  and  $V_L$  domains; His6 tag: sequence encoding six C-terminal histidine residues; hok-sok: plasmid stabilizing DNA locus; lacI: gene encoding lac-repressor; lac P/O: wild-type lac-operon promoter/operator; M13ori: intergenic region of bacteriophage M13; pBR322ori: origin of the DNA replication; PelB leader: signal peptide sequence of the bacterial pectate lyase; rbs1: ribosome binding site derived from E. coli lacZ gene (lacZ); rbs2 and rbs3: ribosome binding site derived from the strongly expressed gene 10 of bacteriophage T7 (T7g10); skp gene: gene encoding bacterial periplasmic factor Skp/OmpH; tHP: strong transcriptional terminator; tLPP: lipoprotein terminator of transcription;  $V_H$  and  $V_L$ : sequence coding for the variable region of the immunoglobulin heavy and light chain, respectively. Unique restriction sites are indicated.

FIGURE 4: Analysis of purified anti-CD3 scFv antibodies by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions

Lane 1:  $M_r$  markers (kDa,  $M_r$  in thousands); Lane 2: anti-CD3 scFv<sub>10</sub>; Lane 3: anti-CD3 scFv<sub>6</sub>. The gel was stained with Coomassie Blue.

FIGURE 5: Analysis of purified anti-CD3 scFv antibodies by size exclusion chromatography on a calibrated Superdex 200 column

The elution positions of molecular mass standards are indicated.

FIGURE 6: Lineweaver-Burk analysis of fluorescence dependence on antibody concentration as determined by flow cytometry

Binding of mAb OKT3 (circles), scFv<sub>6</sub> (triangles) and scFv<sub>10</sub> (squares) to CD3<sup>+</sup> Jurkat cells was measured.

FIGURE 7: Retention of anti-CD3 antibodies on the surface of CD3<sup>+</sup> Jurkat cells at 37°C

Cell-surface retention of mAb OKT3 (circles), scFv<sub>6</sub> (triangles) and scFv<sub>10</sub> (squares) on CD3<sup>+</sup> Jurkat cells was measured. Values are expressed as a percentage of initial mean fluorescence intensity.

FIGURE 8: Proliferation of peripheral blood mononuclear cells (PBMC) after 24 h incubation in presence of mAb OKT3 and anti-CD3 scFv-antibodies at concentrations of 0.01-10 µg/ml

PBMCs from healthy donor A or donor B alone and mixed lymphocyte culture of PBMCs from donor A plus B were seeded in microtiter plates at density of  $2 \times 10^5$  cells/well either without antibodies or in presence of serial dilutions of mAb OKT3, anti-CD3 scFv<sub>6</sub> and anti-CD3 scFv<sub>10</sub>. After 24 h incubation, the cells were pulsed with 10 µM BrdU for 18 h. Incorporation of BrdU was determined by BrdU-ELISA. The means and SDs of triplicates are shown.

FIGURE 9: Proliferation of PBMC after 72 h incubation in presence of mAb OKT3 and anti-CD3 scFv-antibodies at concentrations of 0.01-10 µg/ml

PBMCs from healthy donor A or donor B alone and mixed lymphocyte culture of PBMCs from donor A plus B were seeded in microtiter plates at density of  $2 \times 10^5$  cells/well either without antibodies or in presence of serial dilutions of mAb OKT3, anti-CD3 scFv<sub>6</sub> and anti-CD3 scFv<sub>10</sub>. After 72 h

incubation, the cells were pulsed with 10  $\mu$ M BrdU for 18 h. Incorporation of BrdU was determined by BrdU-ELISA. The means and SDs of triplicates are shown.

FIGURE 10: Release of IL-2 by PBMCs after 24 h incubation in presence of mAb OKT3 and anti-CD3 scFv-antibodies at concentrations of 0.01-10  $\mu$ g/ml

PBMCs from healthy donor A or donor B alone and mixed lymphocyte culture of PBMCs from donor A plus B were seeded in 24-well plates at a density of  $2 \times 10^6$  cells/well either without antibodies or in presence of serial dilutions of mAb OKT3, anti-CD3 scFv<sub>6</sub> and anti-CD3 scFv<sub>10</sub>. After 24 h incubation, samples from the culture supernatants were harvested and the IL-2 concentration was measured by ELISA. The mean values of duplicates are shown.

FIGURE 11: Release of IFN- $\alpha$  by PBMCs after 72 h incubation in presence of mAb OKT3 and anti-CD3 scFv-antibodies at concentrations of 0.01-10  $\mu$ g/ml

PBMCs from healthy donor A or donor B alone and mixed lymphocyte culture of PBMCs from donor A plus B were seeded in 24-well plates at a density of  $2 \times 10^6$  cells/well either without antibodies or in presence of serial dilutions of mAb OKT3, anti-CD3 scFv<sub>6</sub> and anti-CD3 scFv<sub>10</sub>. After 72 h incubation, the samples of culture supernatants were harvested and the concentration of IFN- $\alpha$  was measured by ELISA. The mean values of duplicates are shown.

FIGURE 12: Release of TNF- $\alpha$  by PBMCs after 36 h incubation in the presence of mAb OKT3 and anti-CD3 scFv-antibodies at concentrations of 0.01-0.1  $\mu$ g/ml

PBMCs from healthy donor A or donor B alone and mixed lymphocyte culture of PBMCs from donor A plus B were seeded in 24-well plates at a density of  $2 \times 10^6$  cells/well either without antibodies or in presence 0.1  $\mu$ g/ml and 0.01  $\mu$ g/ml of

mAb OKT3, anti-CD3 scFv<sub>6</sub> and anti-CD3 scFv<sub>10</sub>. After 36 h incubation, samples of the culture supernatants were harvested and the concentration of TNF- $\alpha$  was measured by ELISA. The mean values of duplicates are shown.

FIGURE 13: Induction of the expression of IL-2R $\alpha$  (CD25) on T cells after 90 h incubation of PBMC cultures in presence of mAb OKT3 and anti-CD3 scFv-antibodies at concentrations of 0.01-10  $\mu$ g/ml

PBMCs from healthy donor A or donor B alone and mixed lymphocyte culture of PBMCs from donor A plus B were seeded in 24-well plates at a density of  $2 \times 10^6$  cells/well either without antibodies or in presence of serial dilutions of mAb OKT3, anti-CD3 scFv<sub>6</sub> and anti-CD3 scFv<sub>10</sub>. After 90 h incubation, the CD25 expression was detected by flow cytometry using anti-CD25 mAb B1.49.9. Mean fluorescence intensity values after subtracting background fluorescence are shown.

FIGURE 14: CD3 modulation and coating by mAb OKT3 and anti-CD3 scFv-antibodies

PBMCs from healthy donor A or donor B were seeded in 24-well plates at a density of  $2 \times 10^6$  cells/well either without antibodies or in presence of serial dilutions of mAb OKT3, anti-CD3 scFv<sub>6</sub> and anti-CD3 scFv<sub>10</sub>. After 24 h incubation, the cells were harvested and stained with FITC-conjugated anti-CD3 mAb OKT3, PC5-conjugated anti-TCR $\alpha/\beta$  mAb BMA031. T cells were counterstained with anti-CD5 mAb and analyzed by flow cytometry. Data for CD3 modulation represent the percentage of TCR/CD3 complexes on the surface of treated CD5-positive T cells as a fraction of TCR/CD3 complexes on the surface of untreated CD5-positive T cells. CD3 coating is shown as the fraction of TCR/CD3 complexes which could not be detected by FITC-conjugated OKT3.

FIGURE 15: (a) DNA sequence of plasmid pSKK3-scFv6\_anti-CD3;  
(b) amino acid sequence of the V<sub>H</sub> and V<sub>L</sub> connected by the  
peptide linker SAKTTP encoded by the DNA sequence contained in  
pSKK3-scFv6\_anti-CD3

Thus, the present invention relates to an antibody characterized by the following features:

- (a) it is capable of suppressing an immune reaction;
- (b) it is devoid of constant antibody regions; and
- (c) it binds an epitope on the CD3 complex of the T-cell receptor.

The antibody of the present invention is specific to human TCR/CD3 complex present on all T cells regardless their MHC specificity. Such antibody is capable to suppress the activated T lymphocytes without any significant release of inflammatory cytokines, thus avoiding many of the unpleasant side-effects. The release of cytokines, e.g., IL-2, IFN- $\gamma$  and TNF- $\alpha$  is reduced by a factor more than 100 compared to OKT3. This is in sharp contrast with any known immunosuppressive antibodies. Although immunosuppression can be achieved by the administering such traditional antibodies to humans, their efficacy is often compromised by two factors: the first-dose syndrome resulting from T-cell activation, and the anti-globulin response (e.g. HAMA response) resulting from multiple injections of foreign proteins of non-human origin. The symptoms of antibody toxicity include fever, chills, diarrhea, and vomiting and in severe cases have resulted in death. The syndrome is caused by the release of inflammatory cytokines as result of transient T cell activation. Such activation depends on the interaction of the Fc portion of the antibody and Fc receptors (FcR) on accessory cells to cross-link the CD3 complexes on T cells. The Fc portion of mAbs of murine origin is also the main reason of anti-globulin response. The



antibody of the present invention is devoid of the immunoglobulin constant domains and, therefore, is not able to interact with FcRs and is also much less immunogenic.

The antibodies of the present invention can be prepared by methods known to the person skilled in the art, e.g. by the following methods:

(a) Construction of single chain Fv-antibodies by combining the genes encoding at least two immunoglobulin variable  $V_H$  and  $V_L$  domains, either separated by peptide linkers or by no linkers, into a single genetic construct and expressing it in bacteria or other appropriate expression system.

(b) Non-covalent dimerization or multimerization of single chain Fv-antibodies comprising at least two  $V_H$  and  $V_L$  specific to human CD3 either separated by peptide linkers or by no linkers, in an orientation preventing their intramolecular pairing.

The term "capable of suppressing an immune reaction" means that the antibody is able, on the one hand, to prevent activation of T lymphocytes by foreign alloantigen and, on the other hand, to selectively deplete already activated T cells.

The antibody of the present invention may be a monovalent, bivalent or multivalent antibody.

In a preferred embodiment, the antibody of the present invention is a non-covalent dimer of a single-chain Fv-antibody (scFv) ("diabody"; see Figure 1) comprising CD3-specific  $V_H$  and  $V_L$  domains, either separated by peptide linkers or by no linkers.

In a further preferred embodiment, the antibody of the present invention comprises two single-chain Fv-antibodies (scFv) (see Figure 1) comprising CD3-specific  $V_H$  and  $V_L$  domains.

In a further preferred embodiment, the antibody of the present invention is a single chain diabody (see Figure 1) comprising CD3-specific  $V_H$  and  $V_L$  domains.

The term "Fv-antibody" as used herein relates to an antibody containing variable domains but not constant domains. The term "peptide linker" as used herein relates to any peptide capable of connecting two variable domains with its length depending on the kinds of variable domains to be connected. The peptide linker might contain any amino acid residue, although the amino acid combinations SAKTTP or SAKTTPKLGG are preferred. The peptide linker connecting single scFv of  $(scFv)_2$  and single chain diabodies (scDb) might contain any amino acid residue, although one-to-three repeats of amino acid combination GGGGS are preferred for  $(scFv)_2$  and three-to-four repeats of GGGGS are preferred for scDb.

In a more preferred embodiment, the antibody of the present invention contains variable domains substantially corresponding to the variable domains of the antibody produced by the hybridoma of ATCC deposit number CRL 8001.

In an even more preferred embodiment, the antibody of the present invention is characterized in that a cysteine at position H100A (Kabat numbering system) has been replaced by another amino acid, preferably by a serine.

The present invention also relates to a polynucleotide encoding an antibody of the present invention and vectors, preferably expression vectors containing said polynucleotides. The recombinant vectors can be constructed according to methods well known to the person skilled in the art; see, e.g., Sambrook, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory (1989) N.Y.

A variety of expression vector/host systems may be utilized to contain and express sequences encoding the antibody of the present invention. These include, but are not limited to, microorganisms such as bacteria transformed with recombinant bacteriophage, plasmid, or cosmid DNA expression vectors; yeast transformed with yeast expression vectors; insect cell systems infected with virus expression vectors (e.g., baculovirus); plant cell systems transformed with virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or with bacterial expression vectors (e.g., Ti or pBR322 plasmids); or animal cell systems.

The "control elements" or "regulatory sequences" are those non-translated regions of the vector-enhancers, promoters, 5'- and 3'-untranslated regions which interact with host cellular proteins to carry out transcription and translation. Such elements may vary in their strength and specificity. Depending on the vector system and host utilized, any number of suitable transcription and translation elements, including constitutive and inducible promoters, may be used. For example, when cloning in bacterial systems, inducible promoters such as the hybrid lacZ promoter of the Bluescript.RTM. phagemid (Stratagene, LaJolla, Calif.) or pSport1.TM. plasmid (Gibco BRL) and the like may be used. The baculovirus polyhedrin promoter may be used in insect cells. Promoters or enhancers derived from the genomes of plant cells (e.g., heat shock, RUBISCO; and storage protein genes) or from plant viruses (e.g., viral promoters or leader sequences) may be cloned into the vector. In mammalian cell systems, promoters from mammalian genes or from mammalian viruses are preferable. If it is necessary to generate a cell line that contains multiple copies of the sequence encoding the multivalent multimeric antibody, vectors based on SV40 or EBV may be used with an appropriate selectable marker.

In bacterial systems, a number of expression vectors may be selected depending upon the use intended for the antibody of the present invention. Vectors suitable for use in the present invention include, but are not limited to the pSKK expression vector for expression in bacteria.

In the yeast, *Saccharomyces cerevisiae*, a number of vectors containing constitutive or inducible promoters such as alpha factor, alcohol oxidase, and PGH may be used; for reviews, see Grant et al. (1987) *Methods Enzymol.* 153:516-544.

In cases where plant expression vectors are used, the expression of sequences encoding the antibody of the present invention may be driven by any of a number of promoters. For example, viral promoters such as the 35S and 19S promoters of CaMV may be used alone or in combination with the omega leader sequence from TMV (Takamatsu, N. (1987) *EMBO J.* 6:307-311). Alternatively, plant promoters such as the small subunit of RUBISCO or heat shock promoters may be used (Coruzzi, G. et al. (1984) *EMBO J.* 3:1671-1680; Broglie, R. et al. (1984) *Science* 224:838-843; and Winter, J. et al. (1991) *Results Probl. Cell Differ.* 17:85-105). These constructs can be introduced into plant cells by direct DNA transformation or pathogen-mediated transfection. Such techniques are described in a number of generally available reviews (see, for example, Hobbs, S. and Murry, L. E. in *McGraw Hill Yearbook of Science and Technology* (1992) McGraw Hill, New York, N.Y.; pp. 191-196.

An insect system may also be used to express the antibodies of the present invention. For example, in one such system, *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes in *Spodoptera frugiperda* cells or in *Trichoplusia* larvae. The sequences

encoding said antibodies may be cloned into a non-essential region of the virus, such as the polyhedrin gene, and placed under control of the polyhedrin promoter. Successful insertion of the gene encoding said antibody will render the polyhedrin gene inactive and produce recombinant virus lacking coat protein. The recombinant viruses may then be used to infect, for example, *S. frugiperda* cells or *Trichoplusia* larvae in which APOP may be expressed (Engelhard, E. K. et al. (1994) Proc. Nat. Acad. Sci. 91:3224-3227).

In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, sequences encoding an antibody of the present invention may be ligated into an adenovirus transcription/translation complex consisting of the late promoter and tripartite leader sequence. Insertion in a non-essential E1 or E3 region of the viral genome may be used to obtain a viable virus which is capable of expressing the antibody in infected host cells (Logan, J. and Shenk, T. (1984) Proc. Natl. Acad. Sci. 81:3655-3659). In addition, transcription enhancers, such as the Rous sarcoma virus (RSV) enhancer, may be used to increase expression in mammalian host cells.

Human artificial chromosomes (HACs) may also be employed to deliver larger fragments of DNA than can be contained and expressed in a plasmid. HACs of 6 to 10M are constructed and delivered via conventional delivery methods (liposomes, polycationic amino polymers, or vesicles) for therapeutic purposes.

Specific initiation signals may also be used to achieve more efficient translation of sequences encoding the antibody of the present invention. Such signals include the ATG initiation codon and adjacent sequences. In cases where sequences

encoding the antibody, its initiation codon, and upstream sequences are inserted into the appropriate expression vector, no additional transcriptional or translational control signals may be needed. However, in case where only coding sequence is inserted, exogenous translational control signals including the ATG initiation codon should be provided. Furthermore, the initiation codon should be in the correct reading frame to ensure translation of the entire insert. Exogenous translational elements and initiation codons may be of various origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of enhancers which are appropriate for the particular cell system which is used, such as those described in the literature (Scharf, D. et al. (1994) Results Probl. Cell Differ. 20:125-162).

In addition, a host cell strain may be chosen for its ability to modulate the expression of the inserted sequences or to process the expressed antibody chains in the desired fashion. Post-translational processing which cleaves a "prepro" form of the protein may also be used to facilitate correct insertion, folding and/or function. Different host cells which have specific cellular machinery and characteristic mechanisms for post-translational activities (e.g., CHO, HeLa, MDCK, HEK293, and W138), are available from the American Type Culture Collection (ATCC; Bethesda, Md.) and may be chosen to ensure the correct modification and processing of the foreign antibody chains.

For long-term, high-yield production of recombinant antibodies, stable expression is preferred. For example, cell lines which stably express the antibody may be transformed using expression vectors which may contain viral origins of replication and/or endogenous expression elements and a selectable marker gene on the same or on a separate vector. Following the introduction of the vector, cells may be allowed

to grow for 1-2 days in an enriched media before they are switched to selective media. The purpose of the selectable marker is to confer resistance to selection, and its presence allows growth and recovery of cells which successfully express the introduced sequences. Resistant clones of stably transformed cells may be proliferated using tissue culture techniques appropriate to the cell type.

Any number of selection systems may be used to recover transformed cell lines. These include, but are not limited to, the herpes simplex virus thymidine kinase (Wigler, M. et al. (1977) Cell 11:223-32) and adenine phosphoribosyltransferase (Lowy, I. et al. (1980) Cell 22:817-23) genes which can be employed in tk.sup.- or aprt.sup.- cells, respectively. Also, antimetabolite, antibiotic or herbicide resistance can be used as the basis for selection; for example, dhfr which confers resistance to methotrexate (Wigler, M. et al. (1980) Proc. Natl. Acad. Sci. 77:3567-70); npt, which confers resistance to the aminoglycosides neomycin and G-418 (Colbere-Garapin, F. et al (1981) J. Mol. Biol. 150:1-14) and als or pat, which confer resistance to chlorsulfuron and phosphinotricin acetyltransferase, respectively (Murry, supra). Additional selectable genes have been described, for example, trpB, which allows cells to utilize indole in place of tryptophan, or hisD, which allows cells to utilize histinol in place of histidine (Hartman, S. C. and R. C. Mulligan (1988) Proc. Natl. Acad. Sci. 85:8047-51). Recently, the use of visible markers has gained popularity with such markers as anthocyanins, beta-glucuronidase and its substrate GUS, and luciferase and its substrate luciferin, being widely used not only to identify transformants, but also to quantify the amount of transient or stable protein expression attributable to a specific vector system (Rhodes, C. A. et al. (1995) Methods Mol. Biol. 55:121-131).

A particular preferred expression vector is pSKK3-scFv6\_anti-CD3 deposited with the DSMZ (Deutsche Sammlung für Mikroorganismen und Zellen) according to the Budapest Treaty under DSM 15137 on Aug. 16, 2002.

The present invention also relates to a composition containing an antibody, polynucleotide or an expression vector of the present invention. Preferably, said composition is a pharmaceutical composition preferably combined with a suitable pharmaceutical carrier. Examples of suitable pharmaceutical carriers are well known in the art and include phosphate buffered saline solutions, water, emulsions, such as oil/water emulsions, various types of wetting agents, sterile solutions etc.. Such carriers can be formulated by conventional methods and can be administered to the subject at a suitable dose. Administration of the suitable compositions may be effected by different ways, e.g. by intravenous, intraperitoneal, subcutaneous, intramuscular, topical or intradermal administration. The route of administration, of course, depends on the kind of therapy and the kind of compound contained in the pharmaceutical composition. The dosage regimen will be determined by the attending physician and other clinical factors. As is well known in the medical arts, dosages for any one patient depends on many factors, including the patient's size, body surface area, age, sex, the particular compound to be administered, time and route of administration, the kind of therapy, general health and other drugs being administered concurrently.

A preferred medical use of the compounds of the present invention described above is immunotherapy, preferably a therapy against acute transplant rejections and possibly against autoimmune diseases, such as type I diabetes, multiple sclerosis and rheumatoid arthritis.



The examples below explain the invention in more detail.

**Example 1: Construction of the plasmids pHOG-scFv10/anti-CD3, pHOG-scFv6/anti-CD3, pSKK3-scFv10/anti-CD3 and pSKK3-scFv6/anti-CD3 for the expression of anti-CD3 scFv<sub>10</sub> and scFv<sub>6</sub> antibodies in bacteria**

For constructing the genes encoding the anti-CD3 scFv<sub>10</sub> and scFv<sub>6</sub> (Figure 2), the plasmid pHOG21-dmOKT3 containing the gene for anti-human CD3 scFv<sub>18</sub> (Kipriyanov et al., 1997, Protein Engineering 10, 445-453) was used. To facilitate the cloning procedures, NotI restriction site was introduced into the plasmid pHOG21-dmOKT3 by PCR amplification of scFv<sub>18</sub> gene using primers Bi3sk, 5'-CAGCCGCCCATGGCGCAGGTGCAACTGCAGCAG and Bi9sk, 5'-GAAGATGGATCCAGCGGCCGCGAGTATCAGCCCGGTT. The resulting 776 bp PCR fragment was digested with NcoI and NotI and cloned into the NcoI/NotI-linearized vector pHOG21-CD19 (Kipriyanov et al., 1996, J. Immunol. Methods 196, 51-62), thus generating the plasmid pHOG21-dmOKT3+Not. The gene coding for OKT3 V<sub>H</sub> domain with a Cys-Ser substitution at position 100A according to Kabat numbering scheme (Kipriyanov et al., 1997, Protein Engineering 10, 445-453) was amplified by PCR with primers DP1, 5'-TCACACAGAATTCTTAGATCTATTAAAGAGGAGAAATTAACC and either DP2, 5'-AGCACACGATATCACCGCCAAGCTTGGGTGTTGTTTGGC or OKT\_5, 5'-TATTAAGATATCGGGTGTTGTTTGGCTGAGGAG, to generate the genes for V<sub>H</sub> followed by linkers of 10 and 6 amino acids, respectively (Figure 2). The resulting 507 bp and 494 bp PCR fragments were digested with NcoI and EcoRV and cloned into NcoI/EcoRV-linearized plasmid pHOG21-dmOKT3+Not, thus generating the plasmids pHOG21-scFv10/anti-CD3 and pHOG21-scFv6/anti-CD3, respectively.

To increase the yield of functional scFv-antibodies in the bacterial periplasm, an optimized expression vector pSKK3 was generated (Figure 3). This vector was constructed on the basis of plasmid pHKK (Horn et al., 1996, Appl. Microbiol. Biotechnol. 46, 524-532) containing hok/sok plasmid-free cell suicide system (Thisted et al., 1994, EMBO J. 13, 1960-1968). First, the gene coding for hybrid scFv V<sub>H</sub>3-V<sub>L</sub>19 was amplified by PCR from the plasmid pHOG3-19 (Kipriyanov et al., 1998, Int. J. Cancer 77, 763-772) using the primers 5-NDE, 5'-GATATACATATGAAATACCTATTGCCTACGGC, and 3-AFL, 5'-CGAATTCTTAAGTTAGCACAGGCCTCTAGAGACACAGATCTTTAG. The resulting 921 bp PCR fragment was digested with NdeI and AflIII and cloned into the NdeI/AflIII linearized plasmid pHKK generating the vector pHKK3-19. To delete an extra XbaI site, a fragment of pHKK plasmid containing 3'-terminal part of the lacI gene (encoding the lac repressor), the strong transcriptional terminator tHP and wild-type lac promoter/operator was amplified by PCR using primers 5-NAR, 5'-CACCTGGCGCCCAATACGCAAACCGCC, and 3-NDE, 5'-GGTATTTTCATATGTATATCTCCTTCTTCAGAAATTCGTAATCATGG. The resulting 329 bp DNA fragment was digested with NarI and NdeI and cloned into NarI/NdeI-linearized plasmid pHKK3-19 generating the vector pHKKΔXba. To introduce a gene encoding the Skp/OmpH periplasmic factor for higher recombinant antibody production (Bothmann and Plückthun, 1998, Nat. Biotechnol. 16, 376-380), the skp gene was amplified by PCR with primers skp-3, 5'-CGAATTCTTAAGAAGGAGATATACATATGAAAAAGTGGTTATTAGCTGCAGG and skp-4, 5'-CGAATTCTCGAGCATTATTTAACCTGTTTCAGTACGTCGG using as a template the plasmid pGAH317 (Holck and Kleppe, 1988, Gene 67, 117-124). The resulting 528 bp PCR fragment was digested with AflIII and XhoI and cloned into the AflIII/XhoI digested plasmid pHKKΔXba resulting in the expression plasmid pSKK2. For removing the sequence encoding potentially immunogenic c-myc epitope, the NcoI/XbaI-linearized plasmid pSKK2 was used for cloning the NcoI/XbaI-digested 902 bp PCR fragment encoding

the scFv phOx31E (Marks et al., 1997, BioTechnology 10, 779-783), which was amplified with primers DP1 and His-Xba, 5'-CAGGCCTCTAGATTAGTGATGGTGGTGGTGGTGGG. The resulting plasmid pSKK3 was digested with NcoI and NotI and used as a vector for cloning the genes coding for anti-CD3 scFv<sub>6</sub> and scFv<sub>10</sub>, that were isolated as 715 bp and 727 bp DNA fragments after digestion of plasmids pHOG21-scFv6/anti-CD3 and pHOG21-scFv10/anti-CD3, respectively, with NcoI and NotI.

The generated plasmids pSKK3-scFv6/anti-CD3 (Figure 3) and pSKK3-scFv10/anti-CD3 contain several features that improve plasmid performance and lead to increased accumulation of functional bivalent product in the E. coli periplasm under conditions of both shake-flask cultivation and high cell density fermentation. These are the hok/sok post-segregation killing system, which prevents plasmid loss, strong tandem ribosome-binding sites and a gene encoding the periplasmic factor Skp/OmpH that increases the functional yield of antibody fragments in bacteria. The expression cassette is under the transcriptional control of the wt lac promoter/operator system and includes a short sequence coding for the N-terminal peptide of  $\beta$ -galactosidase (lacZ') with a first rbs derived from the E. coli lacZ gene, followed by genes encoding the scFv-antibody and Skp/OmpH periplasmic factor under the translational control of strong rbs from gene 10 of phage T7 (T7g10). Besides, the gene of scFv-antibody is followed by a nucleotide sequence encoding six histidine residues for both immunodetection and purification of recombinant product by immobilized metal-affinity chromatography (IMAC).

## **Example 2: Production in bacteria and purification of scFv-antibodies**

The *E. coli* K12 strain RV308 ( $\Delta$ lac $\chi$ 74 galISII::OP308strA) (Maurer et al., 1980, J. Mol. Biol. 139, 147-161) (ATCC 31608) was used for functional expression of scFv-antibodies. The bacteria transformed with the expression plasmids pSKK3-scFv6/anti-CD3 and pSKK3-scFv10/anti-CD3, respectively, were grown overnight in 2xYT medium with 100  $\mu$ g/ml ampicillin and 100 mM glucose (2xYT<sub>GA</sub>) at 26°C. The overnight cultures were diluted in fresh 2xYT<sub>GA</sub> medium till optical density at 600 nm (OD<sub>600</sub>) of 0.1 and continued to grow as flask cultures at 26°C with vigorous shaking (180-220 rpm) until OD<sub>600</sub> reached 0.6-0.8. Bacteria were harvested by centrifugation at 5,000 g for 10 min at 20°C and resuspended in the same volume of fresh YTBS medium (2xYT containing 1 M sorbitol, 2.5 mM glycine betaine and 50  $\mu$ g/ml ampicillin). Isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.2 mM and growth was continued at 21°C for 14-16 h. Cells were harvested by centrifugation at 9,000 g for 20 min at 4°C. To isolate soluble periplasmic proteins, the pelleted bacteria were resuspended in 5% of the initial volume of ice-cold 200 mM Tris-HCl, 20% sucrose, 1 mM EDTA, pH 8.0. After 1 h incubation on ice with occasional stirring, the spheroplasts were centrifuged at 30,000 g for 30 min and 4°C leaving the soluble periplasmic extract as the supernatant and spheroplasts plus the insoluble periplasmic material as the pellet. The periplasmic extract was thoroughly dialyzed against 50 mM Tris-HCl, 1 M NaCl, pH 7.0, and used as a starting material for isolating scFv-antibodies. The recombinant product was concentrated by ammonium sulfate precipitation (final concentration 70% of saturation). The protein precipitate was collected by centrifugation (10,000 g, 4°C, 40 min) and dissolved in 10% of the initial volume of 50 mM Tris-HCl, 1 M NaCl, pH 7.0, followed by thorough dialysis

against the same buffer. Immobilized metal affinity chromatography (IMAC) was performed at 4°C using a 5 ml column of Chelating Sepharose (Amersham Pharmacia, Freiburg, Germany) charged with  $\text{Cu}^{2+}$  and equilibrated with 50 mM Tris-HCl, 1 M NaCl, pH 7.0 (start buffer). The sample was loaded by passing the sample over the column by gravity flow. The column was then washed with twenty column volumes of start buffer followed by start buffer containing 50 mM imidazole until the absorbance (280 nm) of the effluent was minimal (about thirty column volumes). Absorbed material was eluted with 50 mM Tris-HCl, 1 M NaCl, 300 mM imidazole, pH 7.0, as 1 ml fractions. The eluted fractions containing recombinant protein were identified by reducing 12% SDS-PAGE followed by Coomassie staining. The positive fractions were pooled and subjected to buffer exchange for 50 mM imidazole-HCl, 50 mM NaCl (pH 7.0) using pre-packed PD-10 columns (Pharmacia Biotech, Freiburg, Germany). The turbidity of protein solution was removed by centrifugation (30,000 g, 1 h, 4°C).

The final purification was achieved by ion-exchange chromatography on a Mono S HR 5/5 column (Amersham Pharmacia, Freiburg, Germany) in 50 mM imidazole-HCl, 50 mM NaCl, pH 7.0, with a linear 0.05-1 M NaCl gradient. The fractions containing scFv-antibody were concentrated with simultaneous buffer exchange for PBS containing 50 mM imidazole, pH 7.0 (PBSI buffer), using Ultrafree-15 centrifugal filter device (Millipore, Eschborn, Germany). Protein concentrations were determined by the Bradford dye-binding assay (Bradford, 1976, Anal. Biochem., 72, 248-254) using the Bio-Rad (Munich, Germany) protein assay kit. SDS-PAGE analysis demonstrated that anti-CD3 scFv<sub>10</sub> and scFv<sub>6</sub> migrated as single bands with a molecular mass ( $M_r$ ) around 30 kDa (Figure 4). Size-exclusion chromatography on a calibrated Superdex 200 HR 10/30 column (Amersham Pharmacia) demonstrated that scFv<sub>6</sub> was mainly in a

dimeric form with  $M_r$  around 60 kDa, while scFv<sub>10</sub> was pure monomer (Figure 5).

### Example 3: Cell binding measurements

The human CD3<sup>+</sup> T-cell leukemia cell line Jurkat was used for flow cytometry experiments. The cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 U/mL penicillin G sodium and 100 µg/ml streptomycin sulfate (all from Invitrogen, Groningen, The Netherlands) at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>.  $1 \times 10^6$  cells were incubated with 0.1 ml phosphate buffered saline (PBS, Invitrogen, Groningen, The Netherlands) supplemented with 2% heat-inactivated fetal calf serum (FCS, Invitrogen, Groningen, The Netherlands) and 0.1% sodium azide (Roth, Karlsruhe, Germany) (referred to as FACS buffer) containing diluted scFv-antibodies or mAb OKT3 (Orthoclone OKT3, Cilag, Sulzbach, Germany) for 45 min on ice. After washing with FACS buffer, the cells were incubated with 0.1 ml of 0.01 mg/ml anti-(His)<sub>6</sub> mouse mAb 13/45/31-2 (Dianova, Hamburg, Germany) in the same buffer for 45 min on ice. After a second washing cycle, the cells were incubated with 0.1 ml of 0.015 mg/ml FITC-conjugated goat anti-mouse IgG (Dianova, Hamburg, Germany) under the same conditions as before. The cells were then washed again and resuspended in 0.5 ml of FACS buffer containing 2 µg/ml propidium iodide (Sigma-Aldrich, Taufkirchen, Germany) to exclude dead cells. The fluorescence of  $1 \times 10^4$  stained cells was measured using a Beckman-Coulter Epics XL flow cytometer (Beckman-Coulter, Krefeld, Germany). Mean fluorescence (F) was calculated using System-II and Expo32 software (Beckman-Coulter, Krefeld, Germany) and the background fluorescence was subtracted. Equilibrium dissociation constants ( $K_d$ ) were determined by fitting the experimental values to the Lineweaver-Burk equation:  $1/F =$

$1/F_{\max} + (K_d/F_{\max})(1/[Ab])$  using the software program PRISM (GraphPad Software, San Diego, CA).

The flow cytometry experiments demonstrated a specific interaction of scFv-antibodies to Jurkat cells expressing CD3 on their surface. The fluorescence intensities obtained for scFv<sub>6</sub> were significantly higher than for scFv<sub>10</sub> reflecting the 10-fold difference in affinity values for these two scFv-antibodies (Figure 6, Table 1). The deduced affinity value for scFv<sub>6</sub> was fairly close to that of mAb OKT3 thus confirming the bivalent binding of scFv<sub>6</sub> to the cell surface.

#### **Example 4: In vitro cell surface retention**

To investigate the biological relevance of the differences between scFv<sub>6</sub>, scFv<sub>10</sub> and OKT3 in direct binding experiments, the in vitro retention of the scFv-antibodies on the surface of CD3<sup>+</sup> Jurkat cells was determined by flow cytometry (Figure 7). Cell surface retention assays were performed at 37°C under conditions preventing internalization of cell surface antigens, as described (Adams et al., 1998, Cancer Res. 58, 485-490), except that the detection of retained scFv-antibodies was performed using mouse anti-(His)<sub>6</sub> mAb 13/45/31-2 (0.01 mg/ml; Dianova, Hamburg, Germany) followed by FITC-conjugated goat anti-mouse IgG (0.015 mg/ml; Dianova, Hamburg, Germany). Kinetic dissociation constant ( $k_{\text{off}}$ ) and half-life ( $t_{1/2}$ ) values for dissociation of antibodies were deduced from a one-phase exponential decay fit of experimental data using the software program PRISM (GraphPad Software, San Diego, CA). The monovalent scFv<sub>10</sub> had a relatively short retention half-life (1.02 min), while scFv<sub>6</sub> and OKT3 had 1.5-fold and 2.5-fold longer  $t_{1/2}$ , respectively, thus correlating well with their higher binding affinities deduced from the direct binding experiments (Figure 7, Table 1).

**Table 1**  
**Affinity and kinetics of anti-CD3 antibodies binding to CD3<sup>+</sup>**  
**Jurkat cells**

Antibody	K <sub>d</sub> (nM)	k <sub>off</sub> (s <sup>-1</sup> /10 <sup>-3</sup> )	t <sub>1/2</sub> (min)
mAb OKT3	2.06	4.47	2.59
scFv <sub>6</sub>	4.58	7.82	1.48
scFv <sub>10</sub>	51.92	11.33	1.02

The dissociation constants (K<sub>d</sub>) were deduced from Lineweaver-Burk plots shown in Figure 6. The k<sub>off</sub> values were deduced from Jurkat cell surface retention experiments shown in Figure 7. The half-life values (t<sub>1/2</sub>) for dissociation of antibody-antigen complexes were deduced from the ratio  $\ln 2/k_{\text{off}}$ .

**Example 5: Isolation of peripheral blood mononuclear cells**  
**(PBMCs)**

Human PBMCs were isolated from the heparinized peripheral blood of healthy volunteers by density gradient centrifugation. The blood samples were twice diluted with PBS (Invitrogen, Groningen, The Netherlands), layered on a cushion of Histopaque-1077 (Sigma-Aldrich, Taufkirchen, Germany) and centrifuged at 800 g for 25 min. The PBMCs located in the interface were collected and washed three times with PBS before use.



**Example 6: Cell proliferation assay**

Isolated PBMCs were resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin G sodium salt and 0.1 mg/ml streptomycin sulfate (all from Invitrogen, Groningen, The Netherlands) and placed to 96-well flat-bottom tissue culture plates (Greiner, Frickenhausen, Germany) at a density of  $2 \times 10^5$  cells per well. Triplicates of cultures were incubated with serial dilutions of soluble antibodies at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> for the indicated time followed by 18 h pulsing with 0.01 mM 5-bromo-2'-deoxyuridine (BrdU). Incorporation of BrdU was determined by Cell Proliferation ELISA (Roche, Mannheim, Germany) according to the manufacturers instructions.

During incubation for 24-36 h, neither scFv<sub>6</sub> nor scFv<sub>10</sub> induced proliferation of both autologous (donor A alone and donor B alone, respectively) and mixed lymphocyte cultures (donor A+B). In contrast, mAb OKT3 demonstrated high mitogenic activity for all tested 24 h cultures, obviously due to CD3-crosslinking via FcγR-bearing cells (Figure 8).

The OKT3-induced T-cell proliferation was significantly higher in autologous PBMC cultures incubated for 72-90 h, while scFv<sub>6</sub> and scFv<sub>10</sub> demonstrated only minor effects in comparison with 24-h incubation (Figure 9). In mixed PBMC cultures (donor A+B) incubated for 72 h without antibody treatment, a mixed lymphocyte reaction (MLR) developed. Treatment of mixed PBMC cultures with OKT3 had no effect on MLR, while both scFv-antibodies were able to suppress MLR in a concentration-dependent manner, thus reaching the background level at a concentration of 10 µg/ml (Figure 9).

**Example 7: Analyses of cytokine release**

For measurement of cytokine secretion by activated lymphocytes,  $2 \times 10^6$  PBMCs were plated in individual wells of 24-well plates (Greiner, Frickenhausen, Germany) in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin G sodium salt and 0.1 mg/ml streptomycin sulfate (all from Invitrogen, Groningen, The Netherlands) together with the indicated antibodies. For determination of secretion of IL-2, TNF- $\alpha$  and IFN- $\gamma$ , aliquots of the culture supernatants were collected after 24 h, 36 h and 72 h, respectively. Cytokine levels were measured in duplicates using the commercially available ELISA kits for IL-2 (Pharmingen, San Diego, CA), TNF- $\alpha$  and IFN- $\gamma$  (Endogen, Cambridge, MA).

In both autologous and mixed PBMC cultures, OKT3 induced a strong release of IL-2 (Figure 10), IFN- $\gamma$  (Figure 11) and TNF- $\alpha$  (Figure 12). In contrast, the autologous PBMC cultures treated with scFv<sub>6</sub> and scFv<sub>10</sub>, respectively, did not produce IL-2 (Figure 10), IFN- $\gamma$  (Figure 11) and TNF- $\alpha$  (Figure 12). Mixed lymphocyte cultures incubated without antibodies demonstrated release of significant amounts of cytokines as a result of allogeneic stimulation. This secretion of IL-2 and IFN- $\gamma$  could be suppressed by scFv-antibodies in a dose-dependent manner (Figures 10 and 11). Bivalent scFv<sub>6</sub> demonstrated approximately tenfold higher efficacy than scFv<sub>10</sub>. In contrast, mAb OKT3 had rather induction than suppression of cytokine release in mixed PBMC cultures.

**Example 8: Alteration of surface antigens on PBMCs treated  
with anti-CD3 antibodies**

For determination the cell surface expression of the alpha-subunit of IL-2 receptor (CD25) as an early activation marker,  $2 \times 10^6$  PBMCs were plated in individual wells of 24-well plates (Greiner, Frickenhausen, Germany) in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin-G sodium salt and 0.1 mg/ml streptomycin sulfate (all from Invitrogen, Groningen, The Netherlands) together with the indicated antibodies. The cells were harvested after 90 h incubation and stained for flow cytometric analysis with PE-conjugated anti-CD25 mAb B1.49.9 and with the corresponding isotype controls (all from Beckman-Coulter, Krefeld, Germany), as described in Example 3.  $10^4$  lymphocytes were analyzed with a Beckman-Coulter Epics XL flow cytometer (Beckman-Coulter, Krefeld, Germany). Mean fluorescence (F) was calculated using System-II software (Beckman-Coulter, Krefeld, Germany), and background fluorescence was subtracted.

PBMCs that were cultured in the presence of OKT3 showed a strong upregulation of the early activation marker IL-2R $\alpha$  (CD25) on their surface, as determined by flow cytometry (Figure 12). In contrast, none of the PBMC cultures treated either with scFv<sub>6</sub> or scFv<sub>10</sub> showed elevated levels of CD25 expression (Figure 13). Thus, these results clearly demonstrate that, unlike mAb OKT3, scFv<sub>6</sub> and scFv<sub>10</sub> do not possess the T-cell activating properties.

**Example 9: Modulation and coating of TCR/CD3 on lymphocytes  
treated with anti-CD3 antibodies**

To measure the modulation and coating of cell surface TCR/CD3 on lymphocytes,  $2 \times 10^6$  PBMCs were plated in individual wells of 24-well plates (Greiner, Frickenhausen, Germany) in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin-G sodium salt and 0.1 mg/ml streptomycin sulfate (all from Invitrogen, Groningen, The Netherlands) together with the indicated antibodies. The cells were harvested after 24 h incubation and stained for flow cytometric analysis with FITC-conjugated OKT3 (Dr. Moldenhauer, German Cancer Research Center, Heidelberg) or PC5-conjugated anti-TCR $\alpha/\beta$  (Beckman-Coulter, Krefeld, Germany) and the corresponding isotype controls (Beckman-Coulter, Krefeld, Germany). The cells were counterstained with anti-CD5 antibodies (Beckman-Coulter, Krefeld, Germany) for T lymphocytes and analyzed with a Beckman-Coulter Epics XL flow cytometer (Beckman-Coulter, Krefeld, Germany). Mean fluorescence (F) of OKT3-FITC and TCR-PC5 from CD5-positive cells was calculated using System-II software (Beckman-Coulter, Krefeld, Germany). Calculation of CD3 modulation and coating was performed as described previously (Cole, M.S. et al., 1997, J. Immunol. 159, 3613-3621):

%CD3 modulation =

$$\frac{\text{untreated cells F(anti-TCR)} - \text{treated cells F(anti-TCR)}}{\text{untreated cells F(anti-TCR)}} \times 100$$

%CD3 coating =

$$\frac{\text{treated cells F(anti-TCR)} - \text{treated cells F(OKT3)}}{\text{control cells F(anti-TCR)} - \text{control cells(OKT3)}} \times 100$$

Coating, which is defined as the number of CD3 molecules on the surface of T lymphocytes that are antibody bound and therefore not detectable by FITC-conjugated mAb OKT3, was only observed in one experiment with the lowest concentration of OKT3 and anti-CD3 scFv<sub>6</sub> (Figure 14). CD3 modulation, which represents the fraction of TCR/CD3 complexes on the surface of T cells that is lost after antibody treatment, is efficiently (>90%) induced by mAb OKT3 and anti-CD3 scFv<sub>6</sub> at concentrations in the range between 0.1 µg/ml and 10 µg/ml (Figure 14). In contrast, the modulation activity of anti-CD3 scFv<sub>10</sub> was much lower and could be observed only at concentrations above 1 µg/ml (Figure 14).